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A Histochemical Change in Myofibers of Sheep Muscle in Refrigeration

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Summary

Muscles sampled immediately after slaughter are used to study composition of myofiber types. This study was designed to ascertain whether muscle samples from refrigerated carcasses are used for the histochemical classification of myofiber types. The activity of myosin ATPase and NADH dehydrogenase and glycogen content in the sheep muscle refrigerated immediately after slaughter was examined by histochemical techniques. Samples from the semitendinosus muscle were stored at 2°C for 24 days and frozen at −23°C for 12 days. The intensity of NADH dehydrogenase activity and glycogen content in myofibers decreased during 1 day of storage, was unchanged from that time to 12 days, and decreased in storage for 24 days. Myofibers were unchanged in NADH dehydrogenase activity but decreased in glycogen content in the frozen and thawed muscle. The reactivity for myosin ATPase in myofibers was unchanged in the refrigerated muscle and frozen-thawed muscle. The results indicate that muscle samples from refrigerated carcasses can be used for the classification of myofiber types from differences in myosin ATPase reaction.

Myofibers in skeletal muscles of domestic animals vary in histochemical activities of NADH dehydrogenase and myosin ATPase (1). A histochemical reactivity for myosin ATPase in myofibers is affected by the pH of the incubation solution before myosin ATPase reaction (2, 3). Myofibers show a weak to strong activity for NADH dehydrogenase (1-3). In the limb muscles of sheep, myofibers are classified into three to four types from differences in the reactivity for myosin ATPase and NADH dehydrogenase (4-6). Histochemical properties of the myofiber types are considered to reflect differences in their physiological function.

Myofibers are broken in refrigerated meat (7, 8). The structure of myofibrils is disintegrated and mitochondira are disrupted in the muscles stored at 2-4°C for 24 hr (9, 10). Such post mortem changes may affect the histochemical activities of myosin ATPase and NADH dehydrogenase in the refrigerated muscle. In frozen meat ice crystals are formed between and within myofibers (11, 12). When

frozen meat is thawed, fluid flows out of the meat: i.e. drip. It is unclear whether the formation of large ice crystals and a reabsorption and outflow of water in the myofibers of the frozen muscle during thawing affect the histochemical reactivity for myosin ATPase and NADH dehydrogenase.

Muscle samples taken from carcasses within 1 hr after slaughter are used routinely for the demonstration of histochemical enzyme activity because the enzyme activity is thought to be changed after death. In the refrigerated muscle of pigs, the histochemical activity of NADH dehydrogenase and myosin ATPase has been reported to be unchanged from time of slaughter until 46 hr (13). This study was designed to clarify a histochemical change in reactivity for myosin ATPase and NADH dehydrogenase activity in myofibers of sheep muscle during refrigeration and in freezing-thawing. A change in glycogen content within myofibers was examined in the refrigerated muscle. If the histochemical activity of NADH dehydrogenase and reactivity for myosin ATPase is preserved during refrigeration, muscles from the refrigerated carcasses are used for the classificaton of myofiber types.

Experimental Procedure

The semitendinosus muscle from a female 2-year-old sheep was used in this study. Eight muscle samples (1 cm along myofibers × 1 cm × 2 cm) were cut off from the muscle within 1 hr after slaughter. One sample was quickly frozen in a mixture of dry ice and acetone after trimming and used as the control (zero day of storage). Five samples out of the remaining samples were put into a petri dish and stored at 2°C. The other two were put into another petri dish and stored at −23°C. The petri dishes were covered, wrapped in plastic wrap, and kept in a refrigerator. The muscle samples stored at 2°C were taken out separately from the petri dish at 1, 3, 6, 12, and 24 days after storage and immediately immersed in the mixture of dry ice and acetone. One of the samples frozen at −23°C for 12 days was taken out from the petri dish and quickly immersed in the mixture of dry ice and acetone. Another one was thawed at room temperature and quickly frozen in the mixture of dry ice and acetone. Cross sections, 10 μm thick, were cut serially on a cryostat. Fresh sections were incubated for the demonstration of myosin ATPase activity (14) after preincubation at pH 4.3 and 10.6 (2, 3) and of NADH dehydrogenase activity (14). Other sections were fixed in Carnoy's fluid for 10 min and stained with periodic acid Schiff (PAS) and hematoxylin for the demonstration of glycogen.

The remainder of the semitendinosus muscle from which the samples were removed was wrapped in plastic wrap, stored in the same refrigerator at 2°C, and used for histological examination. The muscle samples (1.5 cm along myofibers × 1 cm × 0.5 cm) were taken separately from it before storage and at 1, 3, 6, 12, and 24 days after storage. They were fixed in 10% formalin solution and embedded

in paraffin. Longitudinal sections, 4 μm thick, were stained with hematoxylin and eosin.

The optical density of histochemical activity for NADH dehydrogenase and of PAS reaction for glycogen content in myofibers was measured with a microscopic photometer (Olympus MMSP-TR). The wavelength at 640 nm was adopted for measurements of the intensity of NADH dehydrogenase activity and the wavelength at 530 nm for the intensity of PAS reaction. The condenser aperture used for measurements was 6 μm. The peripheral site of myofibers was selected to measure the optical density for NADH dehydrogenase activity because the granules of diformazan deposits were distributed more in the peripheral zone than in the central region of many myofibers (15). The central region of myofibers was selected for measurements of the optical density of PAS reaction because the reaction product was distributed evenly in the myofibers. One hundred myofibers per section were measured at random.

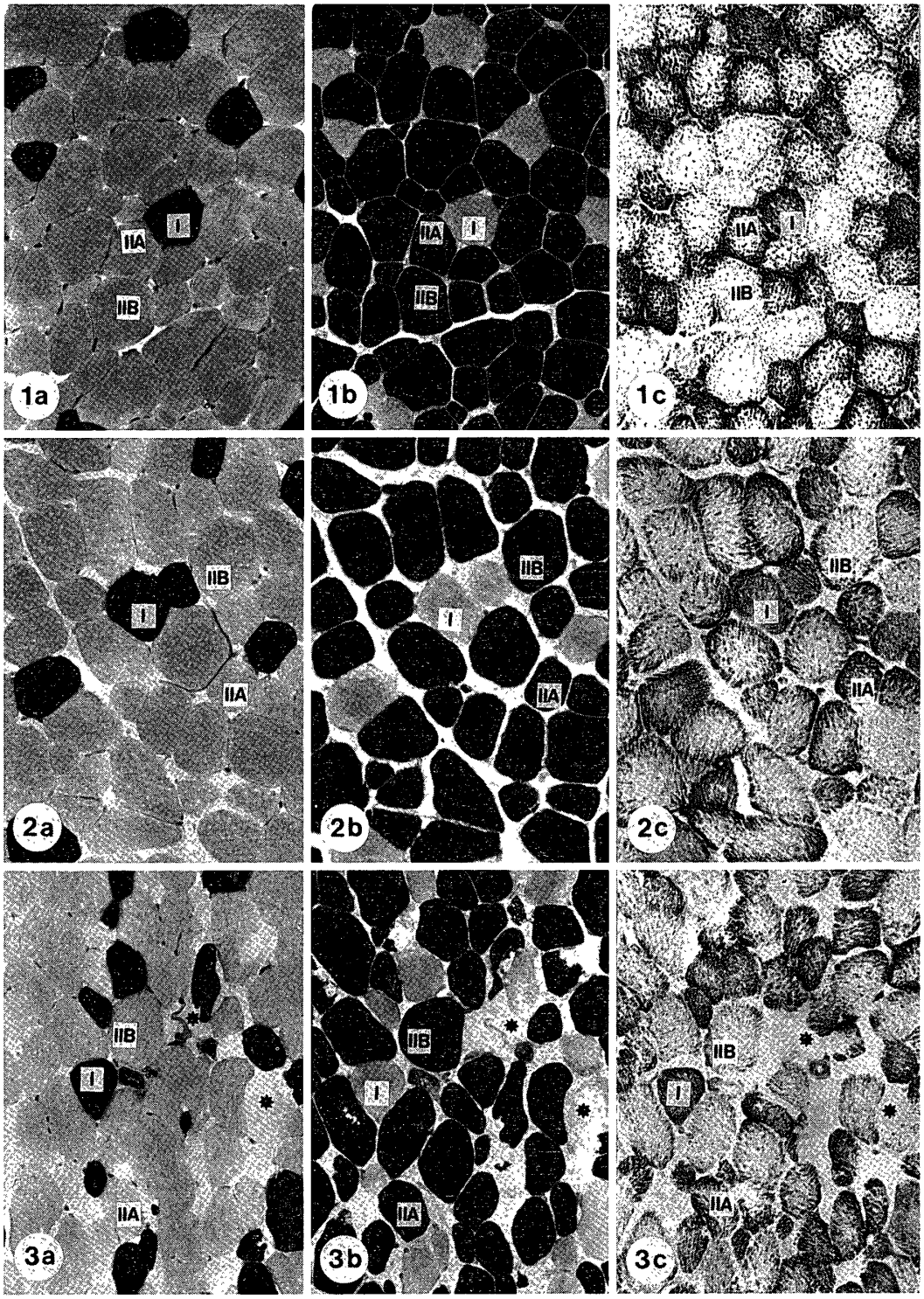
Results

Enzyme activity of refrigerated muscle

In the control and fresh muscle, myofibers were classified into three types (I, IIA, and IIB) as shown in the previous papers (1, 4). Type I myofibers reacted strongly for myosin ATPase after acid preincubation and weakly or negatively for myosin ATPase after alkaline preincubation; they reacted strongly for NADH dehydrogenase (Fig. 1). Type II myofibers reacted negatively for myosin ATPase after acid preincubation and strongly for myosin ATPase after alkaline preincubation. Type II myofibers were classified into type IIA myofibers with a strong activity for NADH dehydrogenase and type IIB myofibers with a weak activity for NADH dehydrogenase (Fig. 1). The diformazan deposits in NADH dehydrogenase activity formed granules in the type IIA and IIB myofibers, and formed open networks or streaks with granules in the type I myofibers.

In refrigeration, the reactivity for myosin ATPase after acid and alkaline preincubation in type I and II myofibers was unchanged in storage for 24 days (Figs. 1-3). The myofibers showing a partial disintegration of myofibrils were found after 3 days of storage, increasing gradually in number from that time on. The disintegration of myofibrils was observed in many myofibers in storage for 24 days (Fig. 3). The reactivity for myosin ATPase was unchanged in the myofibers showing the disintegration of myofibrils (Fig. 3a, b).

In NADH dehydrogenase activity, many type IIA and IIB myofibers showed a streak pattern of diformazan deposits from 1 day through 24 days of storage (Figs. 2c, 3c). The myofibers showed a continuum in the intensity of NADH dehydrogenase activity in the control and stored muscles (Figs. 1c, 2c, 3c). The intensity of NADH dehydrogenase activity in myofibers decreased slightly during 1 day of storage and was unchanged from that time through 12 days of storage



FIGS. 1-3. Histochemical activity of myofibers in the fresh and refrigerated semitendinosus muscle of sheep. FIG. 1. Fresh muscle. FIG. 2. Muscle stored at 2°C for 1 day. FIG. 3. Muscle stored at 2°C for 24 days. Myosin ATPase reaction after preincubation at pH 4.3 (Figs. 1a, 2a, 3a) and at pH 10.6 (Figs. 1b, 2b, 3b) was unchanged, but NADH dehydrogenase activity (Figs. 1c, 2c, 3c) was changed during refrigeration. Asterisks (*) indicate the disappearance of myofibrils. Myofiber types are labeled I, IIA, and IIB. ×160.

TABLE 1. *Histochemical Changes in NADH Dehydrogenase Activity and PAS reaction of Myofibers in Refrigerated and Frozen-thawed Muscle of Sheep*[a]

	Day of storage	NADH dehydrogenase	PAS reaction
Fresh	0	0.301±0.012[b]	0.374±0.009
Refrigerated [c]	1	0.206±0.009	0.195±0.008
	3	0.238±0.012	0.173±0.006
	6	0.208±0.009	0.153±0.006
	12	0.215±0.012	0.205±0.008
	24	0.135±0.007	0.083±0.003
Frozen-thawed[d]	12	0.292±0.013	0.152±0.006

[a] Numerical values indicate the optical density.
[b] Mean±standerd deviation determined from 100 myofibers.
[c] Stored at 2°C
[d] Stored at −23°C

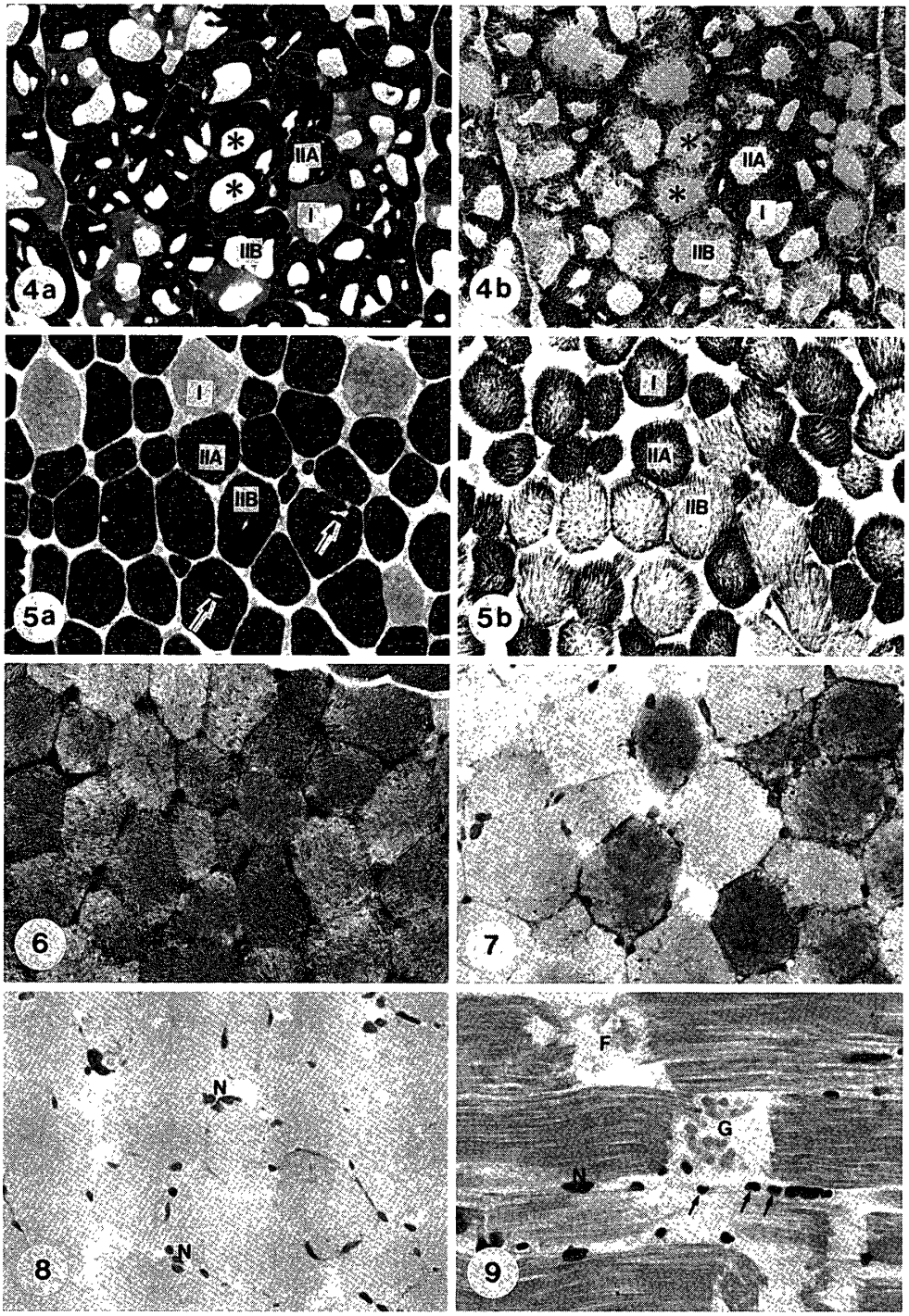
(Table 1). The intensity decreased after 12 days of storage. Although myofibers changed in NADH dehydrogenase activity and in distribution patterns of diformazan deposits in refrigeration, they were classified into type I, IIA, and IIB myofibers by differences in the activity and the reactivity for myosin ATPase.

Enzyme activity of frozen and thawed muscle

In the frozen muscle, ice crystals were formed mostly within myofibers and slightly among the fascicles. The reactivity for myosin ATPase after acid and alkaline preincubation was unchanged in the myofibers in which the myofibrils and sarcoplasm were pressed against the sarcolemma by ice crystals (Fig. 4). The activity of NADH dehydrogenase and the reaction of myosin ATPase and PAS in the myofibers with the distorted and deformed structure appeared to be stronger than that of the myofibers in the fresh muscle. In the frozen-thawed muscle, many myofibers had no gaps formed by ice crystals within them (Fig. 5). Small gaps remained in a few myofibers. The reactivity for myosin ATPase after acid and alkaline preincubation and the intensity of NADH dehydrogenase activity were unchanged in the myofibers of the thawed muscle (Table 1). The distribution pattern of diformazan deposits in myofiber types of the frozen-thawed muscle was similar to that of the refrigerated muscles.

Glycogen content of refrigerated and frozen-thawed muscle

Myofibers of the fresh muscle varied in glycogen content (Fig. 6, Table 1). Some of type IIB myofibers (i.e. white myofibers) were less in glycogen content than type I and IIA myofibers (i.e. red myofibers). Many of the three myofiber types contained much glycogen ; hence, differences in glycogen content among the myofiber types were obscure. Glycogen in myofibers decreased during 1 day of



FIGS. 4 and 5. Histochemical activity of myofibers in the frozen and thawed semitendinosus muscle of sheep. FIG. 4. Muscle frozen at −23°C for 12 days. FIG. 5. Muscle thawed after freezing for the same period of time. The reactivity for myosin ATPase after preincubation at pH 10.6 (Figs. 4a, 5a) and for NADH dehydrogenase (Figs. 4b, 5b) in myofibers was unchanged. Ice crystals (*) form within myofibers in freezing. Small gaps (arrows) formed by ice crystals remain after thawing. Myofiber types are labeled I, IIA, and IIB. ×160.

storage and was unchanged from that time until 12 days of storage (Fig. 7, Table 1). Glycogen decreased greatly and disappeared in numerous myofibers in storage for 24 days (Fig. 8). The myofibers containing no glycogen increased in number in the frozen-thawed muscle, indicating a decrease in glycogen during thawing (Table 1).

Histological changes

In longitudinal sections, myofibers were straight to wavy in the fresh and refrigerated muscle, and showed cross striations. In refrigeration some myofibers showed crinkles. The myofibers showing breaks or ruptures were frequently observed in storage from 6 days onward ; myofibrils were partially disintegrated (Fig. 9). A few myofibers showed breaks at 3 days after storage. No breaks of myofibers were found until 3 days of storage. The structure of nuclei and erthrocytes was preserved in storage for 24 days. The growth of microorganisms was observed on the surface of the muscle sample at 12 days after storage.

Discussion

The reaction product in the histochemical activity of NADH dehydrogenase precipitates as diformazan at the mitochondria (1, 4, 5). The diformazan granules and the reaction product were distributed inhomogeneously within ovine myofibers. The measurements of optical density of the reaction product in the myofibers involve a distributional error (16). In this study, the optical density does not represent an exact intensity of NADH dehydrogenase activity and PAS reaction in the entire myofiber. Nevertheless, the optical density was measured for the demonstration of changes in histochemical activity of NADH dehydrogenase and glycogen content during refrigeration because the measurements of optical density are considered to be more reliable than visual estimation of staining intensity. The optical density of cells and tissues has been shown to be associated with the intenstiy of enzyme activity in them (17).

The breaks in myofibers occur in refrigerated beef (7, 8). Similar changes and disintegration of myofibrils were observed in the ovine muscle stored at 2°C from 3 days onward. The myofibrils disintegrated in the muscles stored at 2-4° C for 24 hr and the mitochondria are swollen and disrupted (9, 10). The slight decreases in NADH dehydrogenase activity during 1 day of storage may be associated with the changes in the structure of mitochondria from the develop-

FIGS. 6-8. Changes in glycogen within myofibers during refrigeration. FIG. 6. Fresh muscle. FIG. 7. Muscle stored at 2°C for 1 day. FIG. 8. Muscle stored at 2°C for 24 days. N : Nuclei. PAS and hematoxylin stain. ×208.

FIG. 9. Breaks of myofibers in the muscle stored at 2°C for 6 days. The disintegration (F) of myofibers and granulation (G) of the sarcoplasm or myofibrils are seen at the sites where myofibrils or myofibers are broken. N : Nuclei. Arrows indicate erthrocytes. Hematoxylin and eosin stain. ×400.

ment of rigor mortis to its resolution. The activity of the enzymes bound to the mitochondria is demonstrated by the histochemical techniques. Myofibrils broke and disappeared at many sites in storage for 24 days. The disintegration of myofibrillar structure must loosen mitochondria from intermyofibrillar spaces. The NADH dehydrogenase activity of the mitochondria that is unfixed within the myofibers with disintegration of myofibrils is not demonstrated histochemically within myofibers. An artificial leakage of mitochondria from the sites where myofibrils disintegrated or lysis of mitochondria might result in decreases in the histochemical activity of NADH dehydrogenase during 24 days of storage.

Glycogen content of myofibers decreased greatly during 1 day of storage. The decreases in glycogen are caused probably by post mortem glycolysis. The pH of muscle lowers after death and reaches the ultimate pH of about 5.5 for 12-24 hr ; the post mortem glycolysis ceases. The decrease in glycogen from 1 day through 24 days of storage may be due to leakage of glycogen from the myofibers with exudate or due to enzymatic degradation of glycogen which is caused by autolysis. In freezing-thawing, the decreases in glycogen seem to be due to leakage of glycogen from myofibers with drip exudate because it is thought that the ultrastructure of the sarcolemma is ruptured by ice crystal formation, and the enzyme is inactive during freezing and thawing.

The type II myofibers of the refrigerated muscles and the frozen-thawed muscle were able to be classified into type IIA and IIB myofibers. However, the proportion of the type IIB myofibers may be greater in the stored muscles than in the fresh muscle because the myofibers classified as type IIB myofibers increase in number owing to decreases in the intensity of NADH dehydrogenase activity. Although the intensity of NADH dehydrogenase activity in the frozen-thawed muscle was unchanged, the distribution of diformazan deposits in the myofibers was deformed as in the myofibers of the refrigerated muscle. The crinkles of myofibers by passive retraction seem to cause the deformation of diformazan deposits. The sections from the refrigerated muscles cannot be used for the classification of type I myofibers into subtype IC and ID myofibers from differences in the distribution pattern of diformazan deposits (5, 6). In the sections from the refrigerated muscles, myofibers were classified clearly into type I and II myofibers because the reaction for acid-stable and alkali-stable myosin ATPase was unchanged during refrigeration. The disintegration of myofibers was greater during 24 days of storage. This study indicates that the muscle sample from sheep carcasses stored at 2°C and at −23°C for 12 days of storage can be used for the classification of myofibers into type I and II myofibers from differences in the reactivity for myosin ATPase.

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